

## EVALUATION OF GENETIC FIDELITY OF *IN VITRO* RAISED PLANTS OF THE IMPORTANT MEDICINAL PLANT HARMAL (*RHAZYA STRICTA* DECNE) USING RAPD AND ISSR MARKERS

EL-AWADY A. M. MOHAMED<sup>1,2</sup>, ELDEESOKY S. DESSOKY<sup>1,3</sup>, ATTIA O. ATTIA<sup>1,3</sup>  
& MOHAMED M. HASSAN<sup>1,4</sup>

<sup>1</sup>Scientific Research Deanship, Biotechnology Research Unit, Taif University, Taif, KSA

<sup>2</sup>Department of Genetics, Faculty of Agriculture, Cairo University, Giza, Egypt

<sup>3</sup>Department of Plant Genetic Transformation, Agricultural Genetic Engineering Research Institute (AGERI),  
Agricultural Research Centre (ARC), Giza, Egypt

<sup>4</sup>Department of Genetics, Faculty of Agriculture, Minufiya University, Sheben El-Kom, Egypt

### ABSTRACT

During the processes of improvement of plant using genetic transformation or the mass production using micropropagation, the most crucial aspect is to retain genetic integrity with respect to the mother plants. In present study, we applied our previously optimized protocol for micropropagation of the important medicinal plant *Rhazya Stricta* Decne for its large scale production. Although no or minor morphological variations were recorded in some micropropagated plants. Randomly Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) markers were employed to determine the genetic fidelity of *R. Stricta* plantlets multiplied *in vitro* by using nodal segments. Among the screened 16 ISSR and 20 RAPD primers, twelve and nine primers, of RAPD and ISSR, respectively, were generated total of 173 clear, distinct and reproducible bands. Among these bands, 164 bands were mono morphic (94.8%) and 9 bands were polymorphic (5.2%). This low polymorphism ration between mother plants and micropropagated plants indicates the little effect of somaclonal variations and the high genetic similarity between mother plants and micropropagated plants. The molecular profiling by using RAPD and ISSR markers proved to be a reliable method for assessing genetic stability of micropropagated plants. To our knowledge, no report was available on the comparative genetic stability of regenerants and mother plant of *R. stricta* by using molecular markers. The developed *in vitro* plants which ascertained stability can be used.

**KEYWORDS:** Medicinal Plant, *Rhazya Stricta*, Genetic Fidelity, RAPD and ISSR Analysis

### INTRODUCTION

Plant tissue culture is recognized as one of the key areas of biotechnology and it can be employed as an alternative to conventional methods of vegetative propagation with the goal of enhancing the rate of multiplication of desired genotypes (Paek and Murthy 2002). Therefore, it has the potential use to regenerate elite and conserve valuable plant genetic resources and scaling up their propagation. Despite the advantages of *in vitro* propagation such as the independent of seasonal variation, mass production, identification and production of clones with desired characteristics, conservation of threatened plant species and production of secondary metabolites, tissue culture is a mutagenic technique causes cytogenetic, genetic and epigenetic variation collectively referred to as tissue culture-induced or somaclonal variation

(Nookaraju and Agrawal 2012). Factors such as growth regulators, time of culture and media composition appear to be the main reasons for inducing somaclonal variations in *In vitro* micropropagated plantlets (Silvarolla, 1992). Therefore, when *in vitro* techniques are used for plant propagation and/or genetic modification the most crucial aspect is to retain genetic integrity with respect to mother plants. Several approaches have been applied for assessment of the genetic purity among micropropagated plants including: phenotypic variation (Kancherla and Bhalla, 2003), cytological studies such as karyotypic analysis of chromosomes (Bennici *et al.*, 2004), biochemical analysis (Punja *et al.*, 2004), field assessment and molecular studies (Devarumath *et al.*, 2007). At present, molecular techniques are powerful and valuable tools used in analysis of genetic fidelity of *in vitro* propagated plants. Several DNA markers have been successfully employed to assess the genomic stability in regenerated plants including those with no obvious phenotypic alternations (Rahman and Rajora 2001). Among the markers, random amplified polymorphic DNA (RAPD) (Williams *et al.*, 1990) and inter simple sequence repeat (ISSR) (Zietkiewicz *et al.*, 1994) have been mostly favored. Simplicity and sensitivity of RAPD (Chuang *et al.*, 2009) and the fact that its polymorphism results from either a nucleotide base change made it useful technique for the analysis of genetic fidelity of many *in vitro* propagated plants. Reports using RAPD technique has successfully been used for the assessment of genetic fidelity in many micropropagated plants (Gheorghe *et al.*, 2009; Khawale *et al.*, 2006; Singh *et al.*, 2005). Inter simple sequence repeats (ISSR) a PCR based genetic marker has been developed as an anonymous, RAPD– like approach that accesses variation in the numerous microsatellite regions dispersed throughout the various genomes and circumvents the challenge of characterizing individual loci that other molecular approaches require. ISSR, system circumvents the requirement for flanking sequence information and thus has found wide applicability in a variety of plants (Srivastava and Gupta, 2008). ISSR technique has successfully been used for the assessment of genetic fidelity in many plants such as *Robina ambigua* (Guo *et al.*, 2006) and the medicinal herb *Swertia chirayita* (Joshi and Dhawan, 2007; Martins *et al.* 2004; Ahmad *et al.*, 2008).

*R. stricta* Decne of the Apocynaceae family is considered one of the most important medicinal plants that grow in the most desert areas in the Arabian Peninsula. It is the most famous plants characterizing the habitat of Saudi Arabia. (Chaudhary and Al- Jowaid, 1999). *R. stricta* shares the vernacular name (Harmal) with another noxious weed, *Peganum harmala* (family Zygophyllaceae). There are two reasons behind the economic value of *R. stricta*. Firstly, it as an invasive weed into rangelands that caused retrogression to their ecological condition. Secondly it is a valuable native medicinal plant that needs to be preserved. The medical benefits of *R. stricta* were proved by many previous studies. It contains milky sap with toxic compounds and many other materials that can be used in the treatment of certain diseases especially as an antimicrobial (Ali, 1998; El-Tarass *et al.*, 2013). Gilani *et al.*, 2007 has identified more than 100 alkaloids that have several pharmacological properties using the Phytochemical analysis of that *R. stricta*. In addition, *R. stricta* is known to be used extensively in folk medicine as an indigenous medicinal herbal drug in treatment of different types of diseases such as skin diseases, stomach diseases, throat sour, in fever, general debility and cancer (Rahman and Qureshi, 1990).

Recently, medicinal plants are widely used for production of many active compounds for herbal and pharmaceutical industries through tissue culture techniques. Earlier, significant efforts have been made for *in vitro* plant micropropagation of *P. harmala* (Saini and Jaiwal, 2000; Ehsanpour and Sa Adat, 2002). However, to date to our best knowledge, our previous study for the *in vitro* multiplication of *R. stricta* Decne was the first to report an improved protocol for micropropagation of this important medicinal plant using nodal segments containing axillary buds (El-Tarras *et al.*, 2012). Thus in the recent study, RAPD and ISSR were employed to assess the genetic similarity in tissue

culture derived plantlets of *R. Stricta*. To the best of our knowledge, this is the very first assessment of somaclonal variation using RAPD and ISSR analyses in the *in vitro* propagated *R. Stricta* regenerants.

## MATERIALS AND METHODS

### Plant Material and Micro Propagation Protocol

Stem nodal contains axillary buds was used for *in vitro* propagation according to our earlier report (El-Tarras *et al.*, 2012). Briefly, nodal contains axillary buds of *R. stricta* were collected from mature plants grown in Taif governorate, KSA. After washing and sterilization of the source tissues, nodal segments were cut into appropriate sizes (1 to 1.5 cm) and cultured on sterile solid basal MS (Murashige and Skoog, 1962) medium gelled with 0.8% (w/v) agar and was supplemented with 2 mg/L BAP and 1 mg/L kin. Breaking of axillary buds and shoot proliferated were observed after 2 weeks. For shoot multiplication, the obtained shoots were subcultures at 4-week intervals on fresh medium with the same compositions. After 4 weeks, the proliferated shoot clusters were sub-cultured in MS medium supplemented with 3 mg/L BAP and 1 mg/L kin for shoot elongation. For roots induction, *in vitro* micro-shoots (2 to 3 cm length) were excised aseptically and implanted on MS basal medium that was solidified with agar and containing 3 mg/L IBA. All the cultures were incubated at  $25 \pm 2^{\circ}\text{C}$  under a 16 h photoperiod with cool and white fluorescent lamps (3000 lux). Plants with good roots were transferred to pots containing a sterile soil and were acclimate in the green house. The complete procedure of *in vitro* propagation of Harmal (*R. stricta*) followed by acclimatization was presented in Figure 1.

### Isolation and Purification of Genomic DNA

Fresh young healthy leaves were collected from the mother and *in vitro* regenerated plants and grounded to powder with liquid  $\text{N}_2$  using a mortar and pestle. Genomic DNA was isolated from leaf samples using the procedure described by the plant isolation kit (Biospin Plant Genomic DNA Extraction Kit, Japan).

**RAPD Analysis:** A mong the total of 20 random primers that were used to detect the polymorphism, the sequence of the 12 primers that produce a clear scorable and reproducible banding pattern is illustrated in (Table 1). The amplification reactions were performed in a 25  $\mu\text{l}$  reaction volume containing about 30 ng genomic DNA, 2mM of each primers (Operon Technologies Inc.), 12.5  $\mu\text{L}$  of Promega master mix (2X) and the final volume was adjusted to 25  $\mu\text{L}$  with PCR water. The PCR reactions were applied using the Bio-Rad C1000 thermal cycler (Germany). An initial step of 5 min at  $94^{\circ}\text{C}$  was performed and followed by 40 cycles of 60 s at  $94^{\circ}\text{C}$ , an annealing step of 1 min at  $37^{\circ}\text{C}$  and an elongation step of 1 min at  $72^{\circ}\text{C}$ ; and finally a 7 min extension at  $72^{\circ}\text{C}$ . The amplification products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide ( $0.5 \mu\text{g mL}^{-1}$ ) in 1X TBE buffer at 95 volts. PCR products were visualized on UV light and photographed using a gel documentation system (Bio-Rad® Gel Doc-2000).

**ISSR Analysis:** A mong the total of 16 ISSR primers that were used to detect the polymorphism, the sequence of the 9 primers that produce a clear scorable and reproducible banding pattern is illustrated in Table 2. PCR reactions were performed in a volume of 20  $\mu\text{l}$  Bio-Rad C1000 thermal cycler (Germany). The amplification reactions were performed in a 25  $\mu\text{l}$  reaction volume containing about 30 ng genomic DNA, 2mM of each primers (Operon Technologies Inc.), 12.5  $\mu\text{L}$  of Promega master mix (2X) and the final volume was adjusted to 25  $\mu\text{L}$  with PCR water. Amplification reactions was  $94^{\circ}\text{C}/5 \text{ min}$ , followed by 30 cycles of  $94^{\circ}\text{C}/1 \text{ min}$ ,  $40\text{--}60^{\circ}\text{C}$  (specific for each primer)/1 min and  $72^{\circ}\text{C}/2 \text{ min}$  and ending with an extension step of  $7^{\circ}\text{C}/7 \text{ min}$ . PCR products were analyzed using agarose (2% w/v) electrophoresis gels stained with ethidium bromide and only bands with high intensity and well separated were selected.

**Data Scoring and Statistical Analysis:** To ensure the absence of artifacts, bands were carefully selected from replicated amplifications (three times). Amplified bands designated by their primer code and their size in base pairs. Data recorded as discrete variables: 1 for the presence and 0 for the absence of a similar band. Only intense and reproducible bands appearing on the gel were scored. Band scoring was analyzed using Gene Tools-gel analysis software of SPSS ver. 16.

## RESULT AND DISCUSSIONS

The assessment of the genetic stability of *in vitro* derived clones is an essential step in the application of biotechnology for micropropagation of true-to-type clones (Eshraghi *et al.*, 2005).

In this study, our previously optimized protocol for micropropagation of the important medicinal plant *Rhiza stricta* (El-Tarras *et al.*, 2012) was used to establish the large scale *in vitro* plants (Figure 1). The micropropagated plants were found to be phenotypically normal and essentially identical with their mother plant at hardening stage which partly suggest the minimal or absence of somaclonal variations.

To test these results at the molecular level, the RAPD and ISSR marker techniques were used to assess the genetic stability of micropropagated plants. Out of 20 and 16 screened primers of RAPD and ISSR, respectively, twelve RAPD and nine ISSR primers produced resolvable, reproducible and scorable bands (Table 1 and Table 2). Total of 173 bands were obtained from the two markers in which 9 bands were polymorphic representing 5.2% polymorphism. In RAPD analysis, twelve primers produced 104 bands with 4.8% polymorphism (5 bands). These five polymorphic bands were produced by only 3 primers (25%). Each of the two primers OPH-01 and OPH-02 produced 2 polymorphic bands that represent 20% and 28.5% of the total bands produced by them, respectively (Figure 2 and table 1) and the primer OPC-02 produced one polymorphic band that represent 11% of the total bands produced by it. The other nine primers (75%) showed 100% monomorphic pattern (The banding pattern produced by the primers OPG-01, OPH-01, OPH-02 and OPD-02 are shown in Figure 2 as an example for these primers). Number of bands produced by the twelve primers was ranged between 7 and 11 with average of 8.7 bands per primers (Table 1). ISSR markers are considered suitable to detect variations among micropropagated plants since a simple sequence repeat targets the fast evolving hypervariable sequences (Rahman and Rajora 2001; Joshi and Dhawan 2007). In ISSR analysis, nine primers produced 69 bands with 5.8% polymorphism (4 bands). Number of bands produced by the nine primers was ranged between 5 and 13 with average of 7.6 bands per primers (Table 2). Each of the four primers ISSR-2, ISSR-4, ISSR-10 and ISSR-11 produced one polymorphic band among the total bands produce by each of them (Table 2). All the bands produced by the five primers ISSR-3, ISSR-5, ISSR-6, ISSR-8 and ISSR-12 was monomorphic (Table 2). Figure 3 is showing ISSR amplification pattern obtained with the four primers ISSR-3, ISSR-4, ISSR-6 and ISSR-11 (with one polymorphic band) and the two primers ISSR-3 and ISSR-6 (with 100% monomorphic pattern).

The high ratio of monomorphic banding pattern in micropropagated and mother plants and the low frequency of polymorphism in RAPD and ISSR profile of micropropagated plantlets (4.8 and 5.8, respectively) suggesting the occurrence of little effects of somaclonal variations and confirms the high genetic homogeneity of the *in vitro* raised plants and also indicates that this micro-propagation protocol is efficient enough to maintain genetic stability. Similarly, Srivastava *et al.* (2006) evaluated < 6% frequency polymorphism of *in vitro* raised sugarcane varieties produced from shoot-tip culture through RAPD markers. Bhowmik *et al.* (2009) reported analysis of genetic uniformity using RAPD markers in micropropagated *Mantisia spathulata*. genetic variation was also detected among *in vitro* regenerated garlic

plants and revealed 20% polymorphism as described by Saker and Sawahel (1998). Joshi and Dhawan (2007) confirmed that the plants of *Swertia chirayita* multiplied through axillary method of micropropagation maintain genetic fidelity even after prolonged period of 168 weeks under *in vitro* condition. Srivastava *et al.* (2006) reported that micropropagated plantlets are often subjected to *in vitro* stress that provokes changes at preferential sites, such as repetitive DNA, thereby activating transposable elements which may cause variation in genetic level due to insertion or deletion. The variation in the RAPD and ISSR pattern can be explained as a result of the insertion/deletion of transposons and point mutation that cause the loss or gain of primer annealing sites (Peschke *et al.* 1991). Employing different marker systems helps in indication of the variation induced by genetic and epigenetic mechanism that likely to be reflected different banding profiles.

The evaluated high genetic similarity of the micropropagated harmal plants raised from nodal segments containing axillary buds with the mother plants in this study support the fact that the axillary multiplication is the safest mode of micropropagation to produce true to type progeny. There are many reports in literature reporting similar results (Carvalho *et al.*, 2004; Martins *et al.*, 2004; Joshi and Dhawan, 2007; Rani and Raina, 2000) also suggested that plants regenerated through organized tissues like meristems or direct somatic embryo-genesis maintain genetic integrity of the plantlets with a least risk of genetic variation.

Similarly, the detection of genetic fidelity in tissue culture raised plant has been reported by many workers using RAPD markers (Rout and Das, 2002; Martins *et al.*, 2004; Venkatachalam *et al.*, 2007) and using ISSR markers (Lopes *et al.*, 2006; Brito *et al.*, 2010; Kumar *et al.*, 2011; Carra *et al.*, 2012; Wang *et al.*, 2012; Nookaraju and Agrawa, 2012). However, the reliability and efficiency of molecular markers are frequently questioned. Therefore, in this study two PCR based techniques, RAPD and ISSR, have been employed to test clonal fidelity because of their simplicity and cost effectiveness. Palombi and Damiano (2002) suggested the use of more than one DNA amplification technique as advantageous in evaluating somaclonal variation while working on micropropagated plants of kiwi fruit. In addition, the usages of two markers that amplify different regions of the genome, allow better chances for the identification of genetic variations in the clones (Martins *et al.*, 2004). The evaluated polymorphism at DNA level in micro-propagated plants which is phenotypically normal in this study was similarly reported before among date palms (Saker *et al.*, 2006).

## CONCLUSIONS

To our knowledge, the present study provides the first report on the genetic fidelity of micropropagated Harmal (*R. stricta*) plants obtained from axillary bud explants using RAPD and ISSR analysis. The obtained results indicated good genetic fidelity obtained in the *in vitro* raised plantlets when examined by the two marker system, hence we concluded that the protocol developed could be effectively used for rapid micropropagation and commercial utilization of *R. stricta* without much risk of genetic instability. Due to high reproducibility and reliability, RAPD and ISSR markers can be efficiently used for determining the genetic homogeneity among tissue culture raised pl

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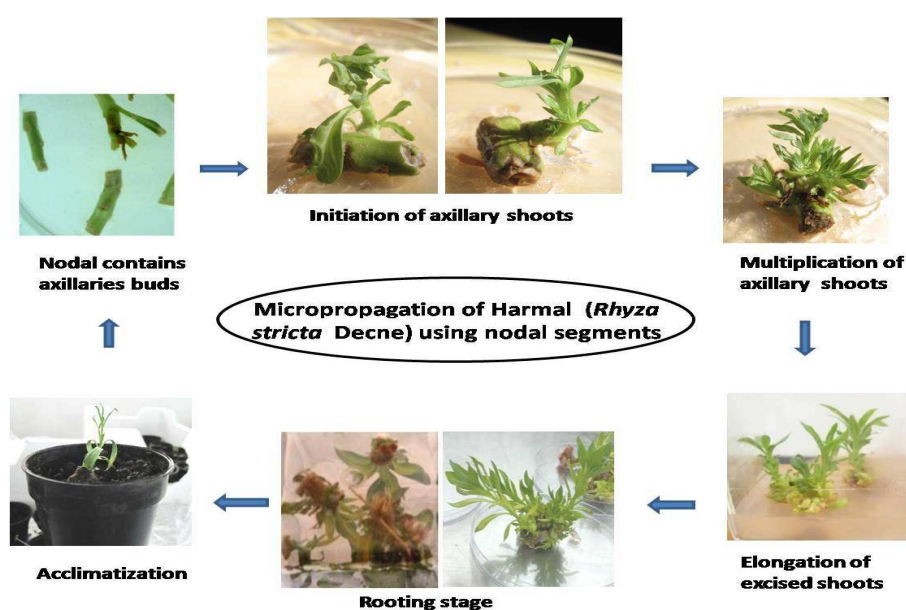
## APPENDICES

**Table 1: List of Random Primers that have been Used for RAPD Analysis, Numbers of Amplicons and %Polymorphism**

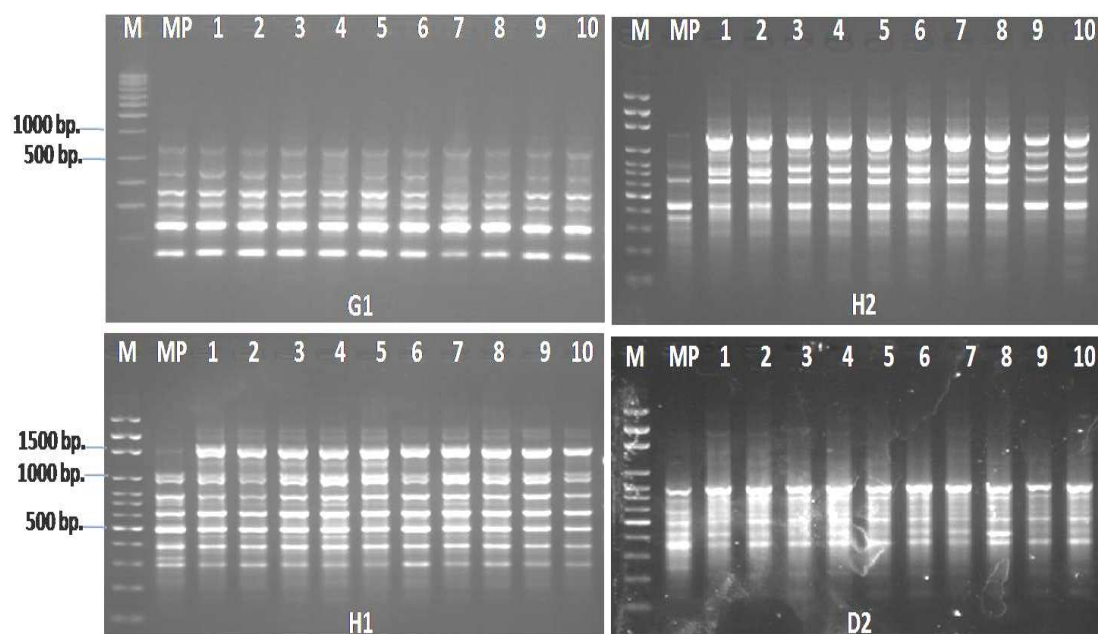
Primer Name	Primer Sequence	No. of Amplicons	No. of Polymorphic Bands	% Polymorphism
OPA-01	5'-CAGGCCCTTC-3'	9	0	0.00
OPA-07	5'-GAAACGGGTG-3'	10	0	0.00
OPA-10	5'-GTGATCGCAG-3'	10	0	0.00
OPB-02	5'-TGATCCCTGG-3'	11	0	0.00
OPC-02	5'-GTGAGGCGTC-3'	9	1	11.0
OPD-02	5'-GGACCCAACC-3'	7	0	0.00
OPE-01	5'-CCCAAGGTCC-3'	8	0	0.00
OPF-10	5'-GGAAGCTTGG-3'	9	0	0.00
OPG-01	5'-CTACGGAGGA-3'	6	0	0.00
OPH-01	5'-GGTCGGAGAA-3'	10	2	20.0
OPH-02	5'-TCGGACGTGA-3'	7	2	28.5
OPH-03	5'-AGACGTCCAC-3'	8	0	0.00
<b>Total</b>		<b>104</b>	<b>5</b>	<b>4.80</b>

**Table 2: List of ISSR Primers that have been Used for ISSR Analysis, Numbers of Amplicons and % Polymorphism**

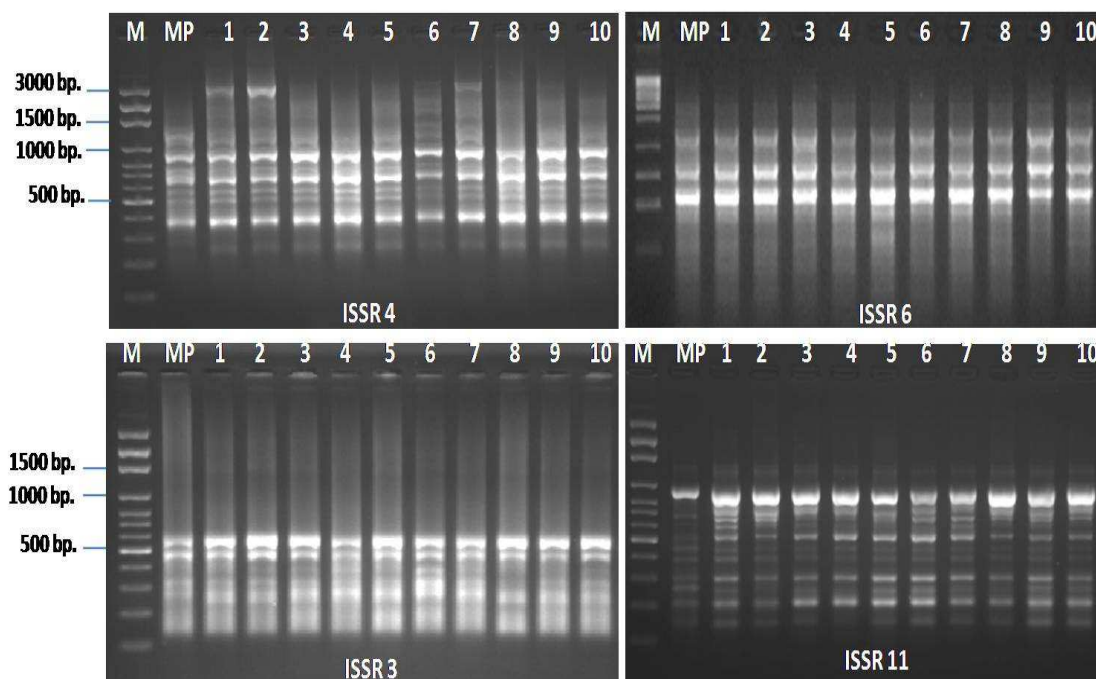
Primer Name	Primer Sequence	No. of Amplicons	No. of polymorphic Bands	% Polymorphism
ISSR-2	(GA)8A	7	1	14.3
ISSR-3	(AG)8YG	6	0	0.00
ISSR-4	(GA)8YT	8	1	12.5
ISSR-5	(GA)8YC	8	0	0.00
ISSR-6	(AC)8YT	5	0	0.00
ISSR-8	BHB(GA)7	6	0	0.00
ISSR-10	BDB(AC)7	11	1	9.00
ISSR-11	(AG)8T	13	1	7.70
ISSR-12	(AG)8C	5	0	0.00
<b>Total</b>		<b>69</b>	<b>4</b>	<b>5.80</b>



**Figure 1: In Vitro Propagation of *Rhazya Stricta Decne* Using Shoot Nodal Segments**



**Figure 2: RAPD Profile of Mother Plant and in Vitro Raised Plants of Harmal (*R. Stricta*) Using Four Random Primers**



**Figure 3: ISSR Profile of Mother Plant and in Vitro Raised Plants of Harmal (*R. Stricta*) Using Four ISSR Primers**